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FLUORESCENT CELL-COUNTING ASSAY OF YELLOW FEVER VIRUS

Nicholas Mahon

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Nicholas Hahon

Aerobiology Division
DIRECTORATE OF BIOLOGICAL RESEARCH

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ABSTRACT

A quantitative assay of infective yellow fever virus particles was developed that is based on the enumeration of cells containing fluorescent viral antigen 24 hours after infection of coverslip McCoy cell cultures. The rapidity and efficiency of virus adsorption onto cell monolayers was markedly enhanced by centrifugation of virus inoculum. By this procedure, 95% of virus was adsorbed within 15 minutes and a proportionality was demonstrated between the number of fluorescent cells and the volume of inoculum.

The incubation period of 24 hours was established from observations on the sequential development of viral antigen within cells and fluorescent cell counts. The relationship between virus concentration and cell-infecting units of virus was linear; the distribution of fluorescent cells in cell monolayers was random. The sensitivity of the fluorescent cell-counting assay was comparable to that of the method of intracerebral inoculation of mice.

A quantitative linear relationship was demonstrated between the quantity of virus neutralized and dilutions of antiserum.

I. INTRODUCTION

Since the original demonstration by Coons et al.[1] that mumps virus antigen may be detected microscopically by staining with fluorescent antibody, the procedure has been successfully employed to visualize viral antigen in cell cultures infected by a wide variety of viruses. Recently, a quantitative assay of infective virus particles was developed that is based on the enumeration of fluorescent cells containing viral antigen. This technique has been standardized for only a limited number of viruses that represent a few major groups, e.g., myxoviruses,[2] adenoviruses,[3] herpesviruses,[4] psittacosis agents,[5] and poxviruses.[6,*] In assays of these viruses, the fluorescent cell-counting technique was shown to be sensitive, precise, reproducible, and rapid (results usually obtained within 24 hours). The usefulness of the technique has been enlarged further with the development of a fluorescent cell-counting neutralization test to detect and to measure quantitatively serum-neutralizing antibody.[7] The feasibility of extending the fluorescent cell-counting technique to the assay of arboviruses was investigated in view of its marked advantages and potential in virus studies.

This report describes the development and standardization of the fluorescent cell-counting technique for the quantitative assay of yellow fever virus.

II. METHODS

A. VIRUS

A plasma suspension of the Asibi strain of yellow fever virus was used throughout this study. Infective plasma was obtained from a rhesus monkey that had been inoculated intraperitoneally with 6×10^4 mouse intracerebral LD_{50} ($MICLD_{50}$) units of virus and bled 3 days later. The plasma was divided into 1-ml portions, sealed in glass vials, and stored at -60 C. The plasma suspension of virus had a titer of $10^{8.1}$ $MICLD_{50}$.

* Unpublished results.

B. CELL LINE AND CULTIVATION

The McCoy cell line was used for the assay of virus. The origin of the cell line, the procedures, and the medium used for cell cultivation have been reported previously.[5] Preliminary tests employing high multiplicities of virus to cells indicated that approximately 99% of the cells were susceptible to infection by yellow fever virus.

C. ROTOR CHAMBER INSERTS

Centrifugation was employed routinely to adsorb virus inoculum onto coverslip cell cultures. To withstand the high centrifugal force required to sediment yellow fever virus, a rotor chamber insert (Figure 1) was designed to fit into the arm of a swinging-bucket type SW 25.1 rotor.* Each rotor chamber insert accommodated a 15-mm coverslip cell culture and as much as 1 ml of inoculum. The chamber insert was sealed with a threaded lid. Two slots in the lid facilitated the removal of the chamber insert with forceps from the arm of the rotor after centrifugation. Each arm of the rotor held four chamber inserts (Figure 2). Rotor chamber inserts were made of Delrin** a plastic that retained its structural integrity after repeated autoclaving for 30-minute intervals at 121 C. Both nylon and Lexan*** polycarbonate resin were also suitable materials.

D. VIRUS ASSAY

Virus dilutions were prepared in maintenance medium consisting of mixture 199 and 5% calf serum and introduced in 0.2-ml volumes directly onto coverslip cell cultures held in sterile rotor chamber inserts. Determinations were generally made in triplicate. Routinely, virus adsorption was carried out in a model L Preparative Ultracentrifuge* at 15,000 rpm (19,642 to 29,432 x g, depending on the distance of the chamber insert in the arm of the rotor from the axis of rotation) for 15 minutes at 25 C. Because the plastic rotor chamber inserts were slightly toxic for cell cultures after prolonged contact, coverslip cell cultures

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* Beckman Instruments, Inc., Spinco Division, Palo Alto, California.
** Kimble Glass Co., Wilmington, Delaware.
*** General Electric Co., Schenectady, N.Y.

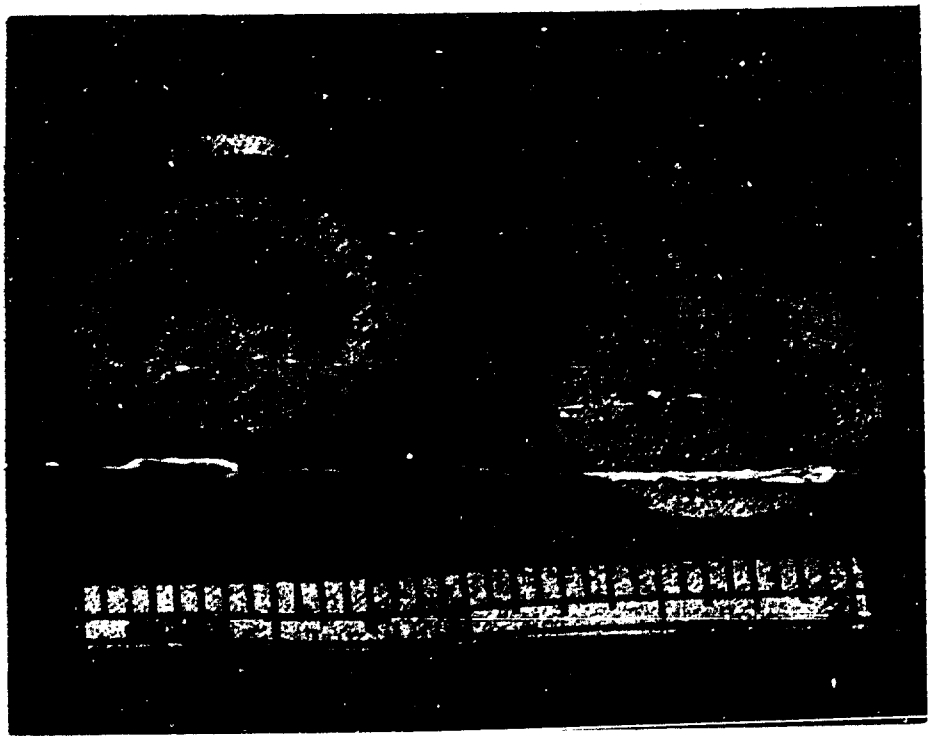

Figure 1. Rotor Chamber Insert for Holding Coverslip Cell Cultures.

Figure 2. Alignment of Rotor Chamber Inserts in Arm of SW 25.1 Swinging-Bucket Rotor.

were removed and inserted into sterile flat-bottomed glass vials (18 x 100 mm). One ml of maintenance medium was added to each vial and the coverslip cell cultures were then incubated at 35 C for 24 hours. Coverslip cell cultures were rinsed twice with cold phosphate buffered saline (PBS), fixed with cold (-60 C) acetone, and either prepared immediately for immunofluorescent staining and cell counting or stored at -60 C. Fixed cell cultures stored at -60 C for as long as 7 weeks showed no appreciable decrease in fluorescence on staining.

E. ANTISERUM

For the preparation of yellow fever virus antiserum, rhesus monkeys were inoculated intraperitoneally with 1 ml of yellow fever vaccine.* Ten days later, the animals were challenged by injection of 1 ml of virulent yellow fever virus. A second challenge dose was administered 2 weeks later; surviving monkeys were bled 2 weeks after the last inoculation. Yellow fever virus antiserum was conjugated with fluorescein isothiocyanate by the method of Riggs et al.[8] The conjugate was adsorbed twice with mouse liver and chick embryo powders.

F. IMMUNOFLUORESCENT PROCEDURES

The direct fluorescent antibody technique was used to obtain immunofluorescence of infected cells. The staining procedure has been described elsewhere.[5] Coverslip cell cultures were examined for fluorescent cells with an American Optical microscope equipped with a Fluorolume illuminator, model 645, Corning no. 5840 and Schott BG-13 exciter filters, and E. K. no. 24 barrier filter. At 430 X magnification with the optical system employed, 1064 microscopic fields were contained in the area of a 15-mm coverslip. Fifty microscopic fields were examined for fluorescent cells in each coverslip cell culture. To calculate the number of cell-infecting units (CIU) of virus per ml, the average number of fluorescent cells per field was multiplied by the number of fields per coverslip, the reciprocal of the dilution of virus inoculum, and a volume factor (for conversion to ml).

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* The National Drug Company, Philadelphia, Pa.

III. RESULTS

A. STANDARDIZATION OF THE FLUORESCENT CELL-COUNTING TECHNIQUE

The efficiency of centrifugation for the adsorption of yellow fever virus onto coverslip cell monolayers was determined at different centrifugal speeds. Virus adsorption was also carried out with inoculated cell cultures maintained in a stationary position. The results in Table 1 show that comparable assay values were obtained with centrifugal speeds of 10,000 to 22,500 rpm. Assay values were significantly lower at centrifugal speeds below 10,000 rpm as well as at stationary incubation.

To determine whether the position of the rotor chamber inserts in fully loaded rotor arms affected the efficiency of virus adsorption onto cell monolayers, virus inoculum was centrifuged at 15,000 rpm for 15 minutes. The centrifugal force from the proximal to the distal chamber insert ranged from 19,642 to 29,430 x g. Results in Table 2 attest to the efficiency of virus adsorption onto coverslip cell cultures in all rotor chamber inserts at the defined conditions of centrifugation. The same experiment was carried out at 10,000 rpm for 15 minutes; the centrifugal force from the proximal to the distal chamber insert ranged from 8730 to 13,080 x g. That adsorption of virus was inefficient at this condition was reflected by a progressive decrease in assay values corresponding to the proximity of the chamber insert to the axis of rotation.

The rate of virus adsorption onto coverslip cell cultures was determined during centrifugation at 15,000 rpm. Virus inoculum was introduced onto coverslip cell cultures held in rotor chamber inserts and centrifuged for designated intervals of time. To determine the quantity of unadsorbed virus, residual inoculum was introduced onto new coverslip cell cultures and centrifuged at 20,000 rpm for 15 minutes. The results indicate that 50% of the virus inoculum was adsorbed in 5 minutes and approximately 95% in 10 minutes. The amount of virus adsorbed during centrifugation periods of 15, 20, and 30 minutes was similar to that of the 10-minute value. In stationarey cultures, 30% of virus inoculum was adsorbed at 35 C for 2 hours and 22% of virus inoculum was adsorbed at 25 C for 4 hours. In view of the results obtained from these experiments, centrifugation at 15,000 rpm for 15 minutes was selected as the condition for routine adsorption of yellow fever virus onto cell monolayers.

TABLE 1. EFFICIENCY OF CENTRIFUGATION VS. STATIONARY INCUBATION ON THE ADSORPTION OF YELLOW FEVER VIRUS ONTO MCCOY CELL MONOLAYERS

Speed,[a] rpm	Centrifugation Centrifugal Force, gravity	CIU[b] per ml
2,000	523	5.3×10^8
3,000	1,177	3.1×10^7
5,000	3,270	5.8×10^7
10,000	13,080	1.1×10^8
15,000	29,430	1.0×10^8
20,000	52,320	1.1×10^8
22,500	65,400	1.0×10^8
	Stationary Incubation	
Temperature, C	Hours	CIU per ml
35	2	4.1×10^7
25	4	3.1×10^7

a. Centrifuged for 15 minutes at 25 C.
b. Cell-infecting units of virus.

TABLE 2. EFFICIENCY OF CENTRIFUGATION ON YELLOW FEVER VIRUS ADSORPTION ONTO MCCOY CELL MONOLAYERS IN ROTOR CHAMBER INSERTS ALIGNED AT DIFFERENT DISTANCES FROM THE AXIS OF ROTATION

Distance from Axis of Rotation, cm	Centrifugal Force,[a] gravity	10^8 CIU[b] per ml
7.8	19,642	1.4
9.1	22,889	1.4
10.4	26,160	1.5
11.7	29,430	1.3

a. Centrifugation at 15,000 rpm for 15 minutes, 25 C.
b. Cell-infecting units of virus.

The efficiency of centrifugation for promoting virus adsorption from different volumes of inoculum is shown in Table 3. The results revealed a proportionality between the number of fluorescent cells and volume of inoculum. No proportionality was noted when similar volumes of inoculum were introduced onto coverslip cell cultures that were then maintained in a stationary position at 35 C for 2 hours. The rate of cell-virus contact appeared to be independent of the volume of inoculum when centrifugal force was employed.

TABLE 3. PROPORTIONALITY BETWEEN VOLUME OF INOCULUM AND CELL-INFECTING UNITS OF YELLOW FEVER VIRUS

Volume, ml	Fluorescent Cells Per 50 Fields	10^6 CIU[a] per ml
0.1	78	1.4
0.2	154	1.5
0.5	360	1.5
1.0	711	1.5

a. Cell-infecting units of virus.

The incubation period, defined here as the interval between virus inoculation and the appearance of recognizable quantities of viral antigen in cell cultures suitable for enumeration of infected cells, was established from sequential observations on the development of viral antigen and fluorescent cell counts. Coverslip cell cultures were inoculated in the prescribed manner and fixed after designated intervals of incubation at 35 C. Within 16 hours after inoculation, a few cells containing faint cytoplasmic fluorescent foci were noted. Fluorescence was more diffuse and granular in appearance at 20 and 22 hours after infection. At 24 and 26 hours, fluorescent cells were easily discerned. Fluorescence was intense and filled the cytoplasm and its elongated extensions (Fig. 3). That a second cycle of infection had occurred was suggested by the presence of varied degrees of fluorescence in cell cultures examined at 30, 44, and 48 hours after inoculation. Some elongated cells contained minute fluorescent foci; others exhibited diffuse fluorescence throughout the cytoplasm. In cells

that had rounded, fluorescence was intense. In general, the development and appearance of fluorescence in infected McCoy cells was similar to that described in infected hamster kidney cells.[9] At 16 to 20 hours, counts of fluorescent cells were one-fifth to one-tenth the uniform counts found at 22 to 26 hours; after this period (30 to 48 hours) they were 3 to 4 times higher. Based on these observations and findings, the optimal period for incubation of cell cultures inoculated with yellow fever virus was established as 24 hours.

Figure 3. Fluorescent Yellow Fever Virus Antigen in McCoy Cells 24 Hours after Infection. Magnification: X 215.

B. QUANTITATIVE EVALUATION OF THE ASSAY

A linear relationship was demonstrated between twofold dilutions of virus over a range of 1.8 log units and the number of cell-infecting units of virus (Fig. 4). These data suggest that each fluorescent cell was the consequence of infection by a single infective virus particle.

In a single experiment, 10 determinations were made to estimate the precision of the fluorescent cell-counting assay for yellow fever virus. Coverslip cell cultures were infected with a standard quantity of virus inoculum and incubated in the prescribed manner. The number of cell-infecting units of virus per ml of inoculum ranged from 1.1×10^8 to 1.6×10^8 with a mean of 1.2×10^8. The standard deviation of 0.18 compared favorably with those obtained in similar studies with the psittacosis agent[6] and variola virus.*

The mode of distribution of fluorescent cells on a coverslip cell monolayer was determined by examing 200 random microscopic fields. The frequencies of fields containing fluorescent cells correspond closely to the theoretical frequencies (Fig. 5). The X^2 test of goodness of fit of the experimental data to the theoretical Poisson distribution gave a probability of approximately 0.75 at d.f. = 8. Fluorescent cells were randomly distributed in infected cell monolayers.

The sensitivity of the fluorescent cell-counting procedure was compared with that of intracerebral inoculation of mice. The results in Table 4 indicate that there was no significant difference in sensitivity between the two assay procedures. Assays showed less variation, however, when performed by the fluorescent cell-counting technique than by the method of intracerebral inoculation of mice.

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* Rahon, unpublished results.

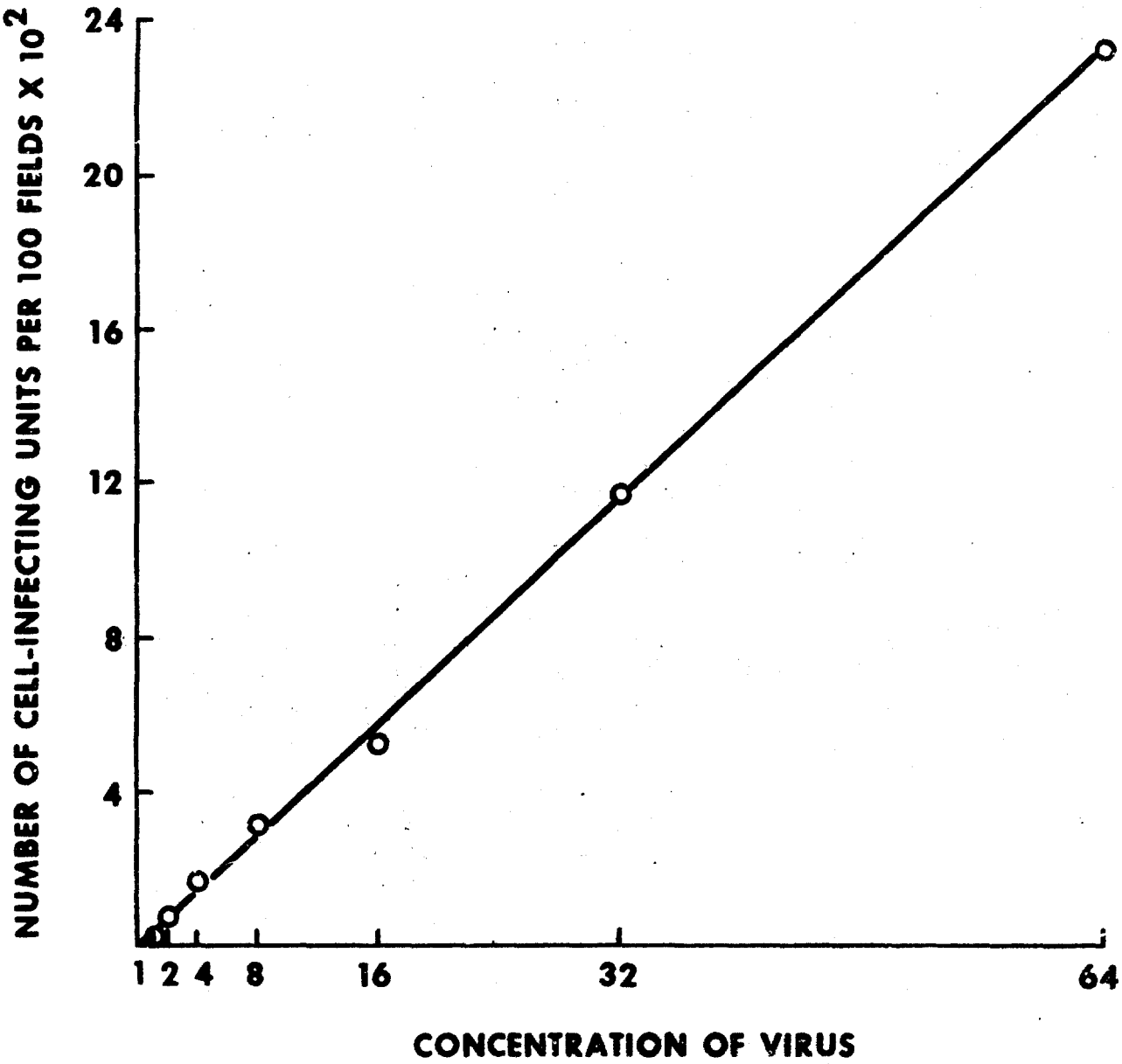

Figure 4. Linear Function Between Concentration of Yellow Fever Virus and the Number of Cell-Infecting Units.

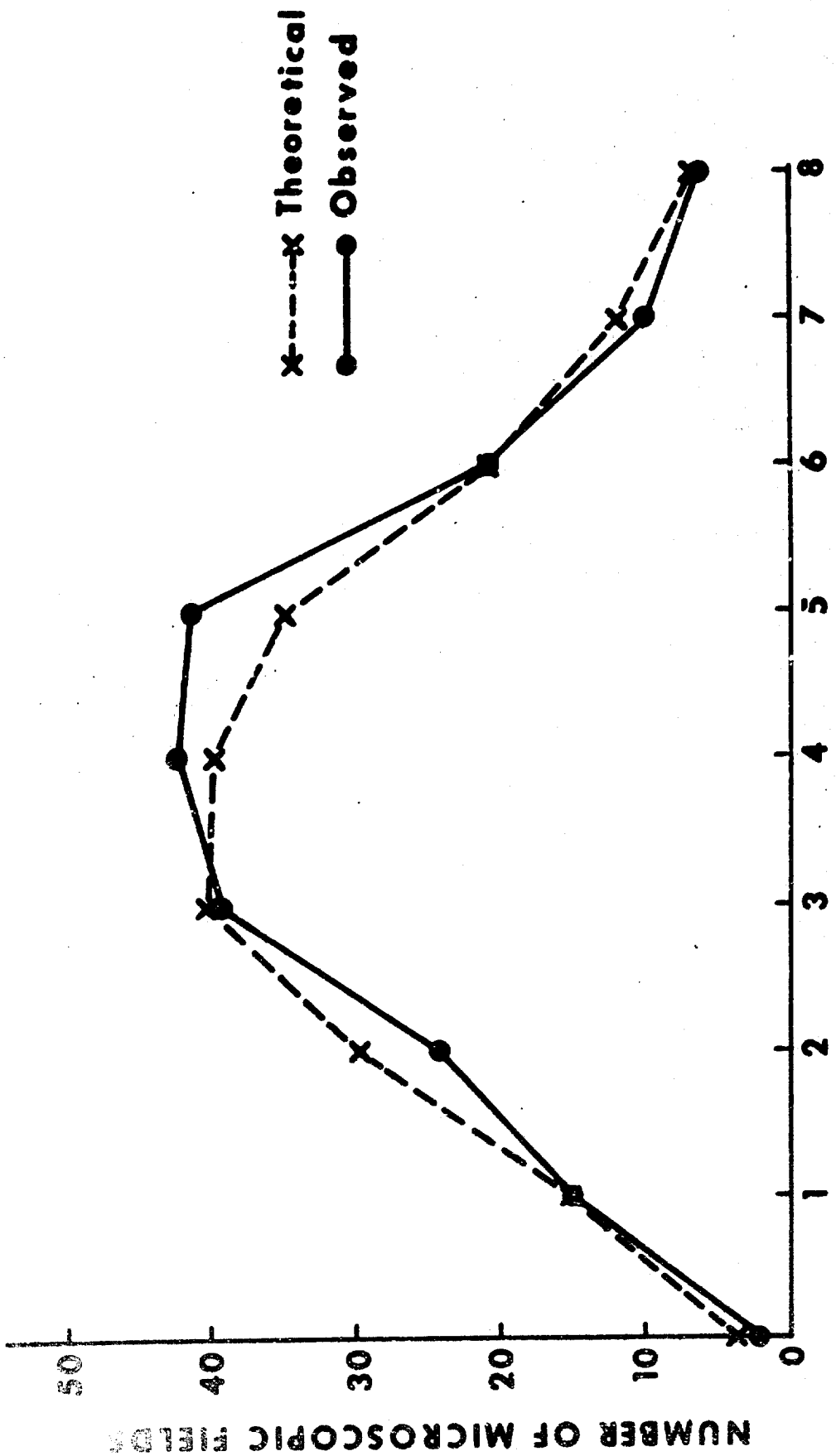

Figure 5. Frequency Distribution of Fluorescent McCoy Cells Infected with Yellow Fever Virus.

TABLE 4. COMPARISON OF TWO PROCEDURES FOR THE ASSAY OF YELLOW FEVER VIRUS

Assay Procedure	Mean	SD	SE, mean
Fluorescent cell-counting	8.1[a]	±0.07	±0.08
Intracerebral inoculation of mice	8.1[b]	±0.67	±0.22

a. Reciprocal of cell-infecting units of virus (log_{10}) per ml based on 10 titrations, determined in 24 hours.
b. Reciprocal of LD_{50} (log_{10}) mouse intracerebral units of virus per ml based on 12 titrations, 14-day observation period.

C. QUANTITATIVE VIRUS-SERUM RELATIONSHIP

Box titrations were carried out by mixing equal volumes of different concentrations of virus suspension and serial twofold dilutions of virus antiserum. Virus-serum mixtures were incubated at 35 C for 1 hour and introduced onto coverslip cell cultures in accord with the standard procedure. A linear function was obtained between the quantity of virus neutralized and antiserum dilutions (Fig. 6). For each 0.5 log unit (3.2-fold) increase of virus concentration, the quantity of antiserum required for neutralization increased approximately 0.3 log unit (2-fold).

IV. DISCUSSION

The use of centrifugal force to promote virus adsorption onto coverslip cell cultures is an important factor that has contributed directly to the successful development of the fluorescent cell-counting technique for the quantitative assay of infectious yellow fever virus particles. The fact that centrifugation of virus inoculum increases the rapidity and efficiency of virus adsorption was previously demonstrated in the development of the fluorescent cell-counting assay for the psittacosis agent[5] and variola virus.* Because of the large size of these infectious particles, a centrifugal force of only 500 x g was required to bring approximately 99% of them in contact with the cell monolayer. With

* Hahon, unpublished results.

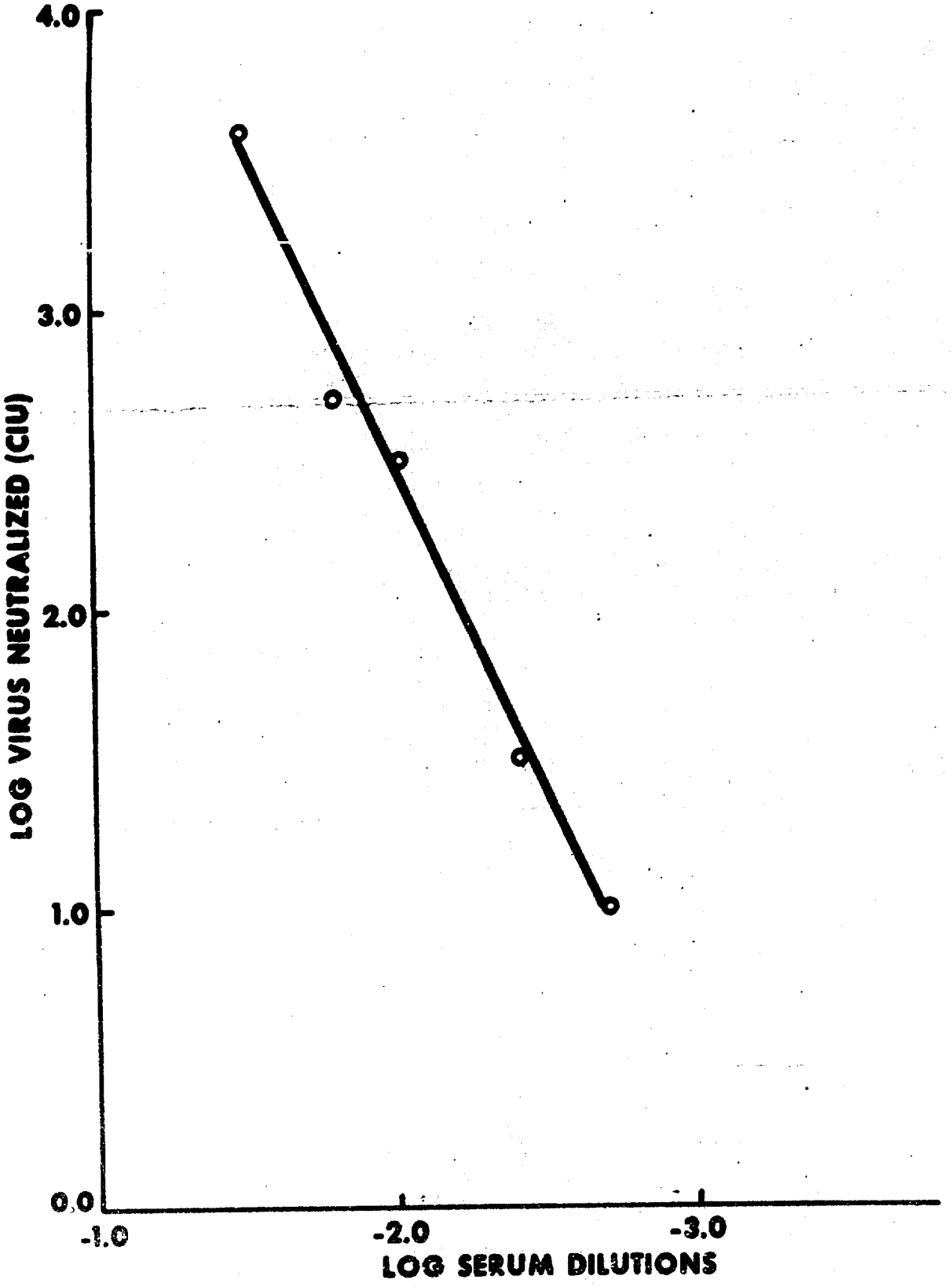

Figure 6. Quantitative Relationship Between the Quantity of Yellow Fever Virus Neutralized and Antiserum Dilutions.

eastern equine encephalomyelitis virus, an infectious agent that approaches the size of yellow fever virus, Gey et al.[10] employed centrifugation at more than 100,000 x g for 1 hour to expose harvested HeLa cell cultures to a suspension of the virus. Evidence that centrifugation enhanced virus adsorption was obtained from the early destructive effect of the virus on cell cultures. Noncentrifuged control cell cultures, exposed to the same amount of virus suspension, showed only the beginning of a cytopathic effect in an equivalent period of time.

The sedimentation and efficient adsorption of yellow fever virus onto coverslip cell monolayers with the aid of high centrifugal speeds was feasible by the use of plastic rotor chamber inserts that were designed to hold coverslips. Approximately 95% of virus was adsorbed from inoculum within 15 minutes at 25 C with centrifugal force that ranged from 19,000 to 29,000 x g, whereas only 30% of virus was adsorbed from inoculated cell cultures held in a stationary position at 35 C for 2 hours. The latter result approximated the findings of Litwin[11] on the adsorption of yellow fever virus in stationary cell cultures held at 35 C for 1 hour. Although only 10 to 20% of the cells could be initially infected with a high inoculum[11] centrifugation of high virus concentrations of inoculum resulted in the infection of approximately 99% of the cells in monolayers. In addition, with the aid of centrifugation, a proportionality was demonstrated between the volume of inoculum and the number of fluorescent cells. The relative efficiency of virus adsorption as a function of the volume of inoculum is relevant to the detection of virus particles from dilute suspensions. Centrifugation of virus inoculum also increased the sensitivity of the fluorescent cell-counting assay so that it was comparable to that of the method of intracerebral inoculation of mice. The advantages of the fluorescent cell-counting technique for the assay of yellow fever virus over that of the latter procedure is in its greater precision, rapidity, specificity, and ease of making replicate determinations.

The concomitant increase in intensity of fluorescent viral antigen in infected cells and the uniformity of fluorescent cell counts at 22 to 26 hours after inoculation served to establish this interval as the length of the incubation period. The initial growth cycle of yellow fever virus lasts 14 to 15 hours after infection, and virus is liberated for at least 10 hours thereafter.[11] Ideally, the incubation period should be terminated before newly synthesized virus particles are released to prevent secondary infection of cells. Because the accumulation of viral antigen in infected cells was insufficient to permit the accurate enumeration of fluorescent cells at 15 hours after inoculation, the incubation period was extended past the time of virus release. This extension period, from the time of virus release (15 hours) to the end of the incubation period (24 hours), did not affect the accuracy of fluorescent cell counts because it was too short to permit the visualization of fluorescent viral antigen in secondarily infected cells.

The demonstration that a quantitative relationship exists between the quantity of yellow fever virus neutralized and dilutions of antiserum provides a basis for the development of a fluorescent cell-counting neutralization test to measure serum-neutralizing antibodies. For research and diagnostic purposes, the advantages of the test would be similar to that offered by the fluorescent cell-counting assay of virus. The assay of yellow fever virus by the procedure described is applicable to the quantitative assay of other arboviruses and those viral agents of small dimensions.

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